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(57) Abstract

The present invention is directed to transgenic animal model systems of Alzheimer's disease (AD). More specifically, the present invention is directed to transgenic non-human mammals over-expressing the α1-antichymotrypsin or β/A4 peptide in brain, and predominantly in neurons and useful test systems for therapeutic and prophylactic interventions in AD. In addition, the present invention is directed to cell lines derived from the transgenic animals of the invention.

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TRANSGENIC ANIMAL MODELS FOR ALZHEIMER'S DISEASE

FIELD OF THE INVENTION

This invention relates to transgenic animal model systems for Alzheimer's disease (AD) which are useful in the study of the etiology and pathogenesis of AD and as systems for testing agents which may alter the course of AD.

BACKGROUND OF THE INVENTION

Alzheimer's disease (AD) is a degenerative neurological disorder characterized by progressive impairment of memory and intellectual function (dementia). Rocca *et al.*, *Ann. Neurol.* **19**:415 (1986). Most patients with AD develop their initial symptoms after 60 years of age and it is one of the most prevalent geriatric health problems in the United States.

There is no effective treatment for the disease and therefore the symptoms of dementia progress inexorably until the death of the patient. In 1987 it was estimated that in the United States alone, an estimated $25 billion per year was being spent on institutional care of demented patients. St. George-Hyslop *et al.*, *Science* **235**:885-890 (1987). It is anticipated that as the average lifespan of the U.S. population continues to increase, the national cost of health care for AD patients will become even more prohibitive and will eventually exceed the amount of the federal budget deficit to become an unsustainable burden on the U.S. health care system. Schneider *et al.*, *J.A.M.A.* **263**:2335-2340 (1990).

The brains of AD patients show characteristic morphological and histological changes including cortical atrophy, loss of neurons, the presence of neurofibrillary tangles, senile neuritic plaques and diffuse amyloid plaques. Neurofibrillary tangles are abnormal neurons in which the cytoplasm is filled with bundles of submicroscopic filamentous structures that are wound around each other in a helical fashion. Katzman, *New Engl. J. Med.* **314**:964-973 (1986). A senile

neuritic plaque is composed of a cluster of degenerating nerve terminals, both dendritic and axonal, with a core that contains extracellular arrays of fine filaments that consist of amyloid protein. Katzman, *New Engl. J. Med.* **314**:964-973 (1986). In over two-thirds of the cases examined, amyloid has been found in blood vessels of the meninges, cerebral cortex, and the hippocampus. Within the brains of patients with AD, as well as patients with Down's Syndrome (who tend to develop full-blown AD type neuropathological symptoms at a relatively early age), diffuse amyloid plaques and the amyloid cores of predominantly neuritic senile plaques contain deposits of the β/A4 peptide. Allsop *et al., Neuropath. Appl. Neurobiol.* **15**:531-542, (1989); Giaccome *et al., Neurosci. Lett.* **97**:232-238 (1989); Tagliavini *et al., Neurosci. Lett.* **93**:191-196 (1988); Masters *et al., Proc. Nat'l. Acad. Sci. USA* **82**:4245-4249 (1985); Glenner *et al., Biochem. Biophys. Res. Comm.* **120**:885-890 (1984); Cras *et al., Proc. Natl. Sci. USA* **88**:7552-7556 (1991); and Paula-Barbosa *et al., J. Neurol. Sci.* **45**:129-134 (1980). In addition to the β/A4 peptide, senile plaques also contain ubiquitin, Hardy *et al., Trends in Pharm. Sci.* **12**:383-388 (1991), the tau protein, Lee *et al., Science* **251**:675-678 (1991), and the protease inhibitor α1-antichymotrypsin (ACT), Abraham *et al., Biotechnology* **7**:147-153 (1989); and Rozemuller *et al., Neurosci. Lett.* **119**:75-78 (1990).

The brain atrophy commonly associated with AD is found primarily in the gyri of the association areas of the cerebral cortex with relative sparing of the primary motor, somatosensory and visual cortices. Terry *et al., Senile Dementia of the Alzheimer type: defining a disease* in *Neurology of Aging*, Katzman *et al* eds., F.A. Davis, Philadelphia, pp. 51-84, (1983).

Cell loss also accompanies Alzheimer's disease, although less than 10% of the neurons in the cerebral hemisphere are lost during the progression of the disease. The major cell groups affected by the disease are neurons with cross-sectional areas larger than $9\mu m^2$. Brains from patients with Alzheimer's show no change in the number of glial cells, the smallest of the cortical cells (less than $40\mu m^2$ in area), although some astrocytes appear to be fibrous. The number of small neurons ($40\mu m^2$ to $90\mu m^2$), the most numerous cells in the cortex, shows no change,

nor is any change seen in the number of cells in the hippocampus, the entorhinal cortex, the locus ceruleus and the nucleus basalis. Katzman, *New Engl. J. Med.* 314:964-973, (1986). It has been suggested that though there is a relatively moderate loss of neurons even in some of the more advanced stages of the disease, that the pattern of cell loss is crucial to the development of the disease. For example, the cells of the entorhinal cortex and hippocampus that are affected by the disease are those which relay messages from other control areas to the hippocampus or which are a major source of output of messages. Thus, the disease process may functionally disconnect the hippocampus with relatively little cell loss. Katzman, *New Engl. J. Med.* 314:964-973 (1986); and Hyman *et al., Science* 225:1168-1170, (1984).

It is believed that amyloid plaques cause the neuronal damage and cell loss that lead to dementia in AD. Hardy *et al., Trends in Pharm. Sci.* 12:383-388 (1991). Interestingly, such plaques are also usually found, albeit in much lower numbers, in the brain of normal elderly subjects. It is thus likely that amyloid plaque formation is a normal sequelae of aging, but that the process is somehow accelerated or exacerbated in AD and in Down's Syndrome.

At present, there is no conclusive evidence that the neurological problems associated with AD result from the effect of any particular type of plaque. Analyses of Down's Syndrome patients who died at different ages suggest that the appearance of diffuse plaques predates the appearance of other typical AD pathological signs such as neurofibrillary tangles and senile neuritic changes. Giaccome *et al., Neurosci. Lett.* 97:232-238 (1989); and Hardy *et al., Trends in Pharm. Sci.* 12:383-388 (1991). These observations have also led to the suggestion that diffuse amyloid plaques can, in specific regions of the brain, lead to the formation of senile plaques and neurofibrillary tangles. Hardy, *et al. Trends in Pharm. Sci.* 12:383-388 (1991).

Recent evidence suggests that the β/A4 peptide can have deleterious neurodegenerative effects on cultured neurons, Yanker *et al., Science* 245:417-420 (1989); and Yanker *et al., Science* 250:279-282 (1990), and when focally injected into rat brain, Frautschy *et al., Neurobiol. Aging* 13:239-253 (1991); and Kowall *et*

al., Proc. Nat'l. Acad. Sci. USA 88:4247-4251 (1991). Furthermore, the latter effect is reportedly reversible by infusions of the neurotransmitter/neuromodulator substance P. Kowall *et al., supra.* Despite these observations, there is still considerable uncertainty as to whether the neurodegenerative effect of the β/A4 peptide, the formation of the amyloid plaque itself, or one or more as yet unidentified events, is the primary cause of the dementia that characterizes AD. Marx, *Science* 257:1336-1338 (1992).

As suggested above, a major component of the plaques seen in AD is the self-aggregating and highly insoluble β-amyloid A4 polypeptide (β/A4) which contains 39 to 43 amino acids and has a molecular weight of approximately 4.2 kD. Glenner, *Cell* 52:307-308 (1988); Glenner *et al., Biochem. Biophys. Res. Comm.* 120:885-890 (1984); and Masters *et al., Proc. Nat'l. Acad. Sci. USA* 82:4245-4249 (1985).

Molecular cloning has revealed that the DNA sequence encoding the β/A4 peptide is embedded within the coding sequence of a much larger amyloid precursor protein (APP) gene located on human chromosome 21. Kang *et al., Nature* 325:733-736 (1987); Goldgaber *et al., Science* 235:887-890 (1987); and Tanzi, *Nature* 331:528-530 (1988). The APP gene is apparently expressed as multiple transcripts, some of which contain an internal coding segment for a Kunitz-type protease inhibitor. Desauvage *et al., Science* 245:651-653 (1989); Kitaguchi *et al., Nature* 331:530-532 (1988); and Ponte, *Nature* 331:525-527 (1988). Transcripts of the APP gene can be found in all tissues of humans, with the highest level of expression found in brain. Bahmanyar *et al., Science* 237:77-88 (1987); and Tanzi *et al., Science* 235:880-884 (1987).

Analyses of the APP gene coding sequences suggest that APP contains features characteristic of glycosylated cell surface receptors. Kang *et al., Nature* 325:733-736 (1987). APP contains a trans-membrane domain and appears to be a cell surface molecule. The β/A4 peptide sequence is located within APP astride the junction of the trans-membrane and extracellular domains. Normally, APP can be cleaved, presumably at the cell surface, to yield secreted forms which are truncated

at the carboxy terminus. Sisodia *et al.*, *Science* **248**:492-495 (1990); and Esch *et al.*, *Science* **248**:1122-1124 (1990). The normal cleavage site is located within the β/A4 peptide sequence, implying that production (via proteolytic cleavage) of these secreted forms of APP could preclude the formation of the β/A4 peptide. These observations suggest the possibility that the β/A4 peptide deposits found in AD represent an abnormal proteolytic product of APP. Cole *et al.*, *Neurochem. Res.* **14**:933-939 (1989); Haass *et al.*, *Nature* **357**:500-503 (1992); and Handy *et al.*, *Science* **256**:184-185 (1992).

Although there is evidence that heredity plays a role in some forms of AD, it has been estimated that up to 95% of all cases of AD occur spontaneously and show no genetic linkage to any single chromosome or gene. Tanzi *et al.*, *J. Biol. Chem.* **266**:20579-20582 (1991). However, two of the rare familial forms of AD have been shown to cosegregate with a mutation of the APP gene itself. Goate *et al.*, *Nature* **349**:704-706 (1991); Murrell *et al.*, *Science* **254**:97-99 (1991); Van Broekhoven *et al.*, *Science* **248**:1120-1122 (1990); and Levy *et al.*, *Science* **248**:1124-1126 (1990). These observations provide evidence supporting the theory that a specific abnormality in amyloid molecules can cause the histopathological features of AD. However, most forms of familialy transmitted AD do not cosegregate with a specific APP allele (or even chromosome #21) among the members of an affected family. Schellenberg *et al.*, *Science* **241**:1507-1510 (1988); and Van Broekhoven *et al.*, *Nature* **329**:153-155 (1987). All indications are that the APP genes of most AD patients contain no mutations in the coding sequences. Thus, a common view is that mutations affecting the structure of this gene are unlikely to be the most common cause of the disease. Glenner, *Cell* **52**:307-308 (1988); Katzman, *New Engl. J. Med.* **314**:964-973 (1986); and St. George-Hyslop *et al.*, *Nature* **347**:194-197 (1990).

There is yet no satisfactory unifying theory that can easily accommodate the observations that (a) some familial forms of AD can be traced to a defective APP gene, (b) most familial forms of familial AD do not cosegregate with genetic transmission of a specific gene allele located on chromosome 21, and (c) most forms of AD do not show a genetically transmitted pattern at all. Yet all patients

suffering from the disease exhibit similar cognitive symptoms and histopathology that coincide with increased numbers of amyloid plaques in the brain.

A recent report by Quon *et al.*, *Nature* 352:239-241 (1992) suggested that overexpression of the APP containing the Kunitz protease inhibitory domain in transgenic mice can result in interstitial amyloid deposits that resemble diffuse amyloid plaques in the human brain. Because of the design of the construct used, it is not possible to ascertain (a) whether the diffuse plaques contain the entire APP protein or only the β/A4 peptide; (b) whether the overexpression of the protease inhibitory domain alone was sufficient to cause amyloid plaque formation; or (c) whether these deposits resulted from overexpression of the β/A4 peptide domain alone, regardless of whether the protease inhibitory domain was present in the transgene.

Recent studies provide support for the theory that abnormal suppression of protease activity in the brain may cause the increased formation of amyloid plaques in the brains of AD patients. Abraham *et al.*, *Cell* 52:487-501 (1988), have shown that one component of AD patients' amyloid deposits in the brain is the serine protease inhibitor α1-antichymotrypsin (ACT). In addition, they showed that significant ACT expression can be detected in regions of Alzheimer's disease patients brains that develop amyloid lesions, but not in the same areas of the brain of aged normal subjects. Overexpression of this protein has previously been observed in some AD patients. Matsubara *et al.*, in *Alzheimer's Disease and Related Disorders*, Iqbal *et al.*, eds. Alan R. Liss, New York (1989).

Rabin *et al.*, *Somat. Cell and Mol. Genet.* 12:209-216 (1986) have mapped the ACT gene to human chromosome 14 and it is interesting to note that one Canadian family displaying a form of inherited Alzheimer's disease showed genetic linkage of the disease with a region of chromosomal 14 in which the α1-antichymotrypsin gene resides. Rabin *et al.*, *supra* and Weitkarp *et al.*, *Am. J. Human Genet.* 35:443-453 (1983). It is possible that an abnormal accumulation of ACT could interfere with the normal proteolytic degradation of APP which would otherwise preclude the formation of the β/A4 polypeptide. The APP thus

accumulated in the brain of a patient with AD could then be degraded via an alternative pathway that could liberate the β/A4 polypeptide which may bind to ACT and form amyloid plaques. Support for the possibility that elevated protease inhibitor expression may lead to increased formation of amyloid deposits comes from the observation that the principal component of the vascular amyloid in the Icelandic form of hereditary cerebral amyloidosis is a variant of the cysteine protease inhibitor cystatin. Ghiso *et al., Proc. Nat'l. Acad. Sci. USA* 83:2974-2978.

Studies of the pathogenesis and treatment of AD have been hampered by the lack of a naturally occurring animal model system for AD. Cell culture systems may be of some utility insofar as investigations into the biochemical and molecular components of AD. However, the ideal model system would be a whole animal system in which the pathophysiological, histopathological, biochemical, and molecular basis as well as the behavioral manifestations of AD may be studied. Additionally, a whole animal system would be particularly useful in assessing various potential treatment modalities for AD and for investigating potential prophylactic measures for the prevention of AD.

Transgenic animals represent one possible approach to the production of an animal model system for AD. Transgenic animals carry a gene which has been introduced into the germline of the animal, or into the germline of an ancestor of the animal, at an early (usually one cell) developmental stage. Three basic approaches have been used to successfully introduce foreign DNA into the germline of animals. The most common involves the direct microinjection of DNA fragments into the pronucleus of a one-cell embryo which is then placed in the oviduct of a foster mother. In another method, cleavage stage embryos are exposed to a recombinant retrovirus *in vitro* after which they are placed into the uterus of a foster mother. Another method involves the growth *in vitro* of pluripotent embryonic stem cells (ES cells) which can be genetically manipulated (by the introducing of foreign DNA fragments) and selected. The genetically altered cells can then be introduced into the embryonic blastocoel after which they can colonize the embryo and ultimately contribute to the germline. See e.g. Merlino, *FASEB J.* 5:2996-3001; (1991).

Transgenic animals have been useful in the study of gene regulation *in vivo*, in the investigation of the role of certain genes in animal development, as models for the investigation of disease and for the study of various approaches to the correction of genetic diseases. Wagner *et al.*, *Proc. Nat. Acad. Sci.* 78, 5016 (1981) and Stewart *et al.*, *Science* 217, 1046 (1982) describe transgenic mice containing human globin genes. Constantini *et al.*, *Nature* 294, 92 (1981) and Lacy *et al.*, *Cell* 34, 343 (1983) describe transgenic mice containing rabbit globin genes. McKnight *et al.*, *Cell* 34, 335 (1983) describes transgenic mice containing the chicken transferrin gene. Brinster *et al.*, *Nature* 306, 332 (1983) describes transgenic mice containing a functionally rearranged immunoglobulin gene. Palmiter *et al.*, *Nature* 300, 611 (1982) describes transgenic mice containing the rat growth hormone gene fused to a heavy metal-inducible metallothionein promoter sequence. Palmiter *et al.*, *Cell* 29, 701 (1982) describes transgenic mice containing a thymidine kinase gene fused to a heavy metal-inducible metallothionein promoter sequence. Palmiter *et al.*, *Science* 222, 809 (1983) describes transgenic mice containing the human growth hormone gene fused to a heavy metal-inducible metallothionein promoter sequence.

Leder *et al.*, U.S. Pat. No. 4,736,866 describes transgenic animals whose germ and somatic cells contain an activated oncogene sequence introduced into the animal at an embryonic stage; U.S. Patent No. 5,175,385 to Wagner *et al.*, teaches transgenic animals which are virus-resistant by virtue of the introduction and expression of a gene coding for human β-interferon into the germ line of a strain of mice; U.S. Patent No. 5,175,384 to Krimpenfort *et al.*, teaches transgenic mice which lack mature T lymphocytes by virtue of the presence of a transgene encoding a T cell receptor polypeptide which inhibits the maturation of T cells.

The development of transgenic animal model systems for human disease represents a particularly promising approach to the understanding of the underlying pathophysiological processes of a disease as well as a promising vehicle for testing new treatments for disease. For example, Kuehn *et al.*, *Nature (London)* **326**:295-298 (1987), and Hooper *et al.*, *Nature (London)* **326**:292-295 (1987), established animal models for Lesch-Nyhan syndrome, a disease involving purine

metabolism which results from a mutation in the hypoxanthine-guanine phosphoribosyl transferase gene. Greaves *et al.*, *Nature (London)* **343**:183-185 (1990) described a transgenic mouse model of sickle cell disorder. Vogel *et al.*, *Nature (London)* **335**:606-611 (1988) showed that the HIV *tat* gene induces lesions resembling Kaposi's sarcoma in transgenic mice. Mullins *et al.*, *Nature (London)* **344**:541-544 (1990) established a model for fulminant hypertension in transgenic rats using the mouse Ren-2 gene. Ohkubo *et al.*, *Proc. Natl. Acad. Sci. USA* **87**:5153-5157 (1990) generated a mouse model for hypertension by introducing the rat renin and angiotensinogen genes into mice. Several other transgenic animal models have also been developed for the study numerous other diseases including neoplasia, eosinophilia, inflammatory disease and others.

U.S. Patent No. 5,175,383 to Leder *et al.*, describes a transgenic animal model for benign prostatic disease. The transgene in these animals comprise an int-2 gene which is operably linked to a promoter effective for the expression of the int-2 gene in the urogenital tissues of the animal. The animal may have utility as a test system for agents suspected of promoting prostatic hyperplasia and as a system for testing procedures for treating or diagnosing benign prostatic hyperplasia or hypertrophy.

Three research groups have described attempts to generate transgenic mouse model systems for AD. However, two of these papers have been retracted.

Wirak *et al.*, *Science* **253**:323-325 (1991), generated two lines of transgenic mice containing a cDNA coding for the human amyloid β protein under the control of the promoter of the human amyloid precursor protein and reported the presence of aggregates of the amyloid β protein in the dendrites of some but not all hippocampal neurons in 1-year-old transgenic mice. However, Jucker *et al.*, *Science* **255**:1443-1445 (1992) showed that the strain of mice used in the study (C57BL/6) show similar amyloid type deposits in the absence of the transgene. This report and additional data of their own prompted Wirak *et al. Science* **255**:1445 (1992) to state that their data "cannot be used to support the conclusion that the β/A4 transgene used in the study can cause formation of amyloid-like deposits in mouse brain". Jucker

et al., do suggest that it is possible that even though expression of the transgene is low, that it may influence the development or composition of the spontaneously occurring deposits.

Kawabata *et al.*, *Nature* **354**:476-478 (1991) attempted to establish a mouse model system for AD in which the transgenic mice contain a transgene consisting of 3.6 kb of 5′ flanking DNA from the human Thy-1 gene (which is expressed in the human central nervous system) linked to a DNA fragment encoding the C terminus of the amyloid precursor protein. The authors' noted expression of RNA from the transgene on embryonic day 15 which increased to stable high levels by two months of age, and asserted that RNA expression was specific to the brain as shown by *in situ* hybridization. The authors also claimed that histological studies showed diffuse amyloid deposits and neurofibrillary tangles in the brains of the transgenic mice as visualized by staining with anti-β/A4 specific antibodies, with silver stain, and by thioflavin staining. However, the authors subsequently retracted the paper because they were unable to reproduce the histopathological findings described in their paper. Kawabata *et al.*, *Nature* **356**:23 (1992).

Quon *et al.*, *Nature* **352**:239-241 (1991) prepared transgenic mice which are asserted to express β APP751 (amyloid precursor protein) under the control of a promoter from rat neuron-specific enolase which has been shown to direct the neuron-specific expression of β-galactosidase in transgenic mice. The transgenic animals described in this study show overexpression of the APP751 which allegedly results in the formation of diffuse interstitial amyloid deposits. However, the data in Quon do not allow the determination of whether or not the diffuse plaques seen in the transgenic animals contain the entire APP protein (or only the β/A4 peptide), whether an overexpression of the protease inhibitory domain alone was sufficient to cause amyloid plaque formation, or whether the deposits result from overexpression of the β/A4 peptide domain alone regardless of whether the protease inhibitory domain was present in the transgene.

Three abstracts directed to animal model systems for AD were presented at the *Third International Conference on Alzheimer's Disease*, (July, 1992).

Nalbontoglu *et al.*, in Abst. No. 397, *supra* reported the generation transgenic mice using a transgene comprising a cDNA fragment encoding amino acids 591-695 of the β-amyloid protein cloned into the first exon of the human neurofilament (NF-L) gene under control of the NF-L promoter. Northern blot analysis of RNA from transgenic lines generated using this transgene suggested that three out of seven lines had transgene derived transcripts. In addition, the authors suggest that transgenic animals have spatial learning deficits as tested using the Morris water maze task.

Greenberg *et al.*, Abst. No. 398, *supra* discussed the use of several promoter systems in attempts to express various segments of APP-695 (an amyloid precursor protein). The authors maintain that the beta protein domain (amino acids 593-695) was expressed under the control of a chimeric metallothionein-growth hormone promoter system with the APP sequences replacing the growth hormone coding sequence [see, Swansen *et al.*, *Nature* 367:363 (1985)] and claim that the transgene was expressed in pyramidal neurons within the cortical and hippocampal regions of brain at levels up to six-fold above endogenous APP RNA. Based on immunohistological data, the authors also suggest that transgene expression leads to elevated accumulation of endogenous APP in these brain regions.

Mucke *et al.*, Abst. No. 399, *supra*, discussed the expression of mutated and non-mutated forms of human APP in the brains of transgenic mice. Transgene constructs used in these studies comprised four human APPs (APP 695, APP 751, and mutated APP 695, and 751 carrying a Val to Ile mutation found in some cases of familial AD) fused with a vector derived from the neuron-specific enolase gene. The authors assertedly detected expression of these four constructs based on Northern and Western blot analysis and by immunostaining of brain sections using human APP antibody. In addition, the authors describe a transgene construct comprising a human ACT cDNA fused with a vector from the murine glial fibrillary acidic protein gene (GFAP) and claim that the GFAP vector effectively directs the inducible and astrocyte-specific expression of hybrid genes *in vivo*. Six separate lines of GFAP-ACT containing mice were said to have been established but no data

describing expression of the transgene construct in animals or whether these animals showed any brain histopathology were reported in the abstract.

Of interest to the background of the invention is Salbaum *et al.*, U.S. Patent No. 5,151,503, which describes the cloning of a promoter region for DNA sequences which encode the precursor of the human A4 amyloid protein. Features of the promoter include multiple RNA start sites, the absence of a typical TATA box and high GC content of the DNA upstream of the RNA start site, placing the amyloid protein promoter in the class of promoters of "housekeeping" genes like the adenosine deaminase gene and the dihydrofolate reductase gene. The promoter sequence was shown to direct expression of a chloramphenicol acetyl-transferase gene when transfected into cells in culture. The patent also suggests that the promoter may be useful in the production of transgenic animals.

There remains a need for a well-defined, reproducible animal model system of AD which exhibits the biochemical and genetic processes implicated in the development of AD. Such a model would also be useful for studies directed toward the prevention, treatment and diagnosis of Alzheimer's disease.

SUMMARY OF THE INVENTION

The present invention is directed to a transgenic non-human mammal all of whose germ cells and somatic cells contain a transgene introduced into the mammal or an ancestor of the mammal. The transgenes of the present invention comprise regulatory sequences and a coding sequence. The regulatory sequences of the transgene are capable of directing predominantly neuronal expression of the coding sequence in the brains of transgenic animals. The coding sequences of the present invention are selected from the group consisting of sequences coding for the β/A4 peptide and the human α1-antichymotrypsin (ACT). Preferred regulatory sequences of the present invention comprise a 2.7 kb upstream sequence of the murine adenosine deaminase gene (ADA) and the SV40 polyadenylation sequence.

Presently preferred transgenes for use according to the present invention include a first transgene construct comprising a 2.7 kb upstream regulatory

sequence of the murine ADA gene located upstream (5′) to an amyloid precursor protein (APP) leader sequence which in turn is fused to the 5′ end of the β/A4 peptide coding region, which lies 5′ to an SV40 polyadenylation sequence (β/A4 transgene construct). Another transgene construct useful in the present invention comprises the 2.7 kb upstream regulatory sequence from the murine ADA gene located upstream (5′) to the entire coding sequence of the human α1-antichymotrypsin gene which in turn lies 5′ to the SV40 polyadenylation sequence (ACT transgene construct).

The present invention is also directed to an autonomously replicating plasmid comprising the β/A4 transgene construct of the present invention.

In addition, the present invention is directed to an autonomously replicating plasmid comprising the ACT construct of the present invention.

The present invention is also directed to the use of other transgenes which code for proteins (such as those containing Kunitz protease inhibitor sequences) which may alter the normal proteolytic processing of the amyloid precursor protein.

Another aspect of the present invention is directed to test systems using the transgenic animals of the present invention for the testing of potential therapies for the treatment or prevention of Alzheimer's disease.

The present invention is also directed to the establishment of cell culture systems using cells derived from the transgenic animals of the present invention, or by transfection with the transgene constructs of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 graphically illustrates the β/A4-containing transgene present in plasmid pADAβAP+L1;

Figure 2 graphically illustrates the ACT-containing transgene present in plasmid pADAhACT;

Figure 3 presents a Southern blot analysis of DNA from transgenic animals containing the ACT transgene;

Figure 4 presents a Southern blot analysis of DNA from transgenic animals containing the β/A4 containing transgene;

Figure 4 presents an agarose gel showing the results of reverse transcriptase polymerase chain reaction analysis of RNA from the brains of animals containing the ACT transgene;

Figure 6 presents photomicrographs of *in situ* hybridization analysis of the expression of the ACT transgene in transgenic mouse brain (Panels A-C) and from a wild type mouse (Panel D);

Figure 7 presents photomicrographs of silver stained brain sections from transgenic mice expressing the ACT transgene (Panels A-E), and from a control mouse lacking the ACT transgene (Panel F);

Figure 8 presents photomicrographs of immunolabeled α1-antichymotrypsin containing lesions in the brain of a transgenic mouse expressing the human ACT gene (Panels A-D) and a human patient with AD (Panel E-F); and

Figure 9 (Panels A-C) presents photomicrographs of immunolabeled β/A4 containing lesions in the brain of the transgenic mouse expressing the human ACT gene.

DETAILED DESCRIPTION OF THE INVENTION

The general strategy employed in the generation of the transgenic animal models for AD was to construct transgenes for the targeted expression of the β/A4 peptide and ACT in the brain of transgenic animals. Both transgenes were constructed using a pTZ19U (U.S. Biochemicals, Cleveland Ohio) based expression vector as described by Rauth *et al., Somat. Cell Genet.* **16**:129-141 (1990), which comprises a 2.7 kb upstream fragment of the murine adenosine deaminase gene (ADA) which was shown to direct the expression of a downstream gene in the brains of transgenic mice. The transgene construct comprising the β/A4 peptide coding sequences also included a sequence coding for the 17 amino acid APP leader sequence fused to the 5′ end of the β/A4 peptide coding sequence. The transgene construct comprising the human ACT gene including its own leader sequence also contained the

2.7 kb upstream sequence from the mouse ADA gene located 5′ to the ACT coding sequence. Both constructs contained an SV 40 polyadenylation sequence located 3′ to the respective coding sequences.

Transgenic mice were generated by microinjection of the appropriate transgene into single-cell mouse embryos according to the method described by Ross *et al., Proc. Nat'l. Acad. Sci. USA* 82:5880-5884 (1985). Potential founder mice were then analyzed for the presence of the transgene by Southern blot analysis. Founder mice were tested for their ability to transmit the transgene to their offspring by breeding to non-transgenic mice. Transgenic offspring were identified using Southern blot analysis. Transgenic offspring of the founder mice were then analyzed for expression of the transgenes in brain by *in situ* hybridization (for the ACT transgene) and by reverse transcriptase-polymerase chain reaction. Finally, transgenic offspring were analyzed by histologic and immunohistologic techniques for the presence of protein deposits resulting from over-expression of the respective transgenes.

Example 1

Construction of pADAβAP+L1

The plasmid pADAβAP+L1 (Figure 1) was deposited with the American Type Culture Collection on March 25, 1993 and has been assigned the Accession Number ATCC 69270.

The transgene construct containing the β amyloid peptide (β/A4) sequence was prepared by synthesizing DNA fragments corresponding to the 17 amino acid leader and 43 amino acid β-amyloid peptide (β/A4) portions of the human amyloid precursor protein (APP), in such a way that the 3′ end of the leader sequence overlapped with the 5′ end of the gene fragment encoding the β/A4 peptide. Kang *et al., Nature* 325:733-736 (1987). The two fragments were then be annealed and extended using the splicing by overlap extension (SOE) method described by Horton *et al., BioTechniques* 8:528-535 (1990) to create a new artificial gene encoding a single

polypeptide comprising the leader sequence of APP fused to the β/A4 peptide with all of the intervening sequences, and all of the downstream sequences, deleted.

The APP leader sequence was synthesized as overlapping oligonucleotides, which were then annealed to each other and filled in under PCR conditions described below, and then joined to a β/A4 peptide sequence obtained initially by SOE. The fused product was then cloned into the expression vector described above and in Rauth *et al., supra.*

For the initial amplification of the APP leader sequences, the primer sequences used were designed based on Kang *et al., supra* and were modified to incorporate a Bam HI cloning site, and three redundant bases at 5′ end of each primer, overlap to allow joining to β/A4 region. The sequences used were as follows:

βAP A: 5′ATAGGATCCAGTCTGCCCGGTTTGGCACT 3′; (SEQ ID NO. 1); and

βAP B: 5′ATTCTGCATCCGCCCGAGCCGTCCAGGCGG 3′; (SEQ ID NO. 2).

For the initial amplification of the β amyloid peptide encoding sequences, primer sequences used were:

βAP C: 5′GGCTCGGGCGGATGCAGAATTCCGACATGA 3′; (SEQ ID NO. 3) and

βAP D: 5′ATAGGATCCCTATGACAACACCGCCCACC 3′; (SEQ. ID NO. 4)

PCR was carried out in a 100 μl reaction volume containing 2 units of Taq DNA polymerase (Promega Biotech, Madison, Wisconsin) (DNA) in a 1.5 mM $MgCl_2$, 0.2 mM dNTPs (dATP, dTTP, dCTP and dGTP), reaction buffer containing 50 μl of non-purified DNA from heat denatured phage stock (from a λgt10 human testis cDNA library) and 10 pmol of each of primer βAP A and βAP B. Amplification was carried out, after incubating for 5 min. at 94°, via 35 cycles of (1)

1 min. at 94°; (2) 1 min. at 60°; (3) 1 min. at 72°; followed by 10 min. at 72°; and storage at 4°,

Bands of the appropriate size (60 bp for the leader sequence and 140 bp for the β/A4 segment) were isolated from a 3% agarose/TBE gel stained with 0.5 μg/ml EtBr, and DNA was isolated using a 0.45 μm spin filter (Millipore, Bedford, MA). The recovered DNA was ethanol precipitated, resuspended, annealed, and extended under PCR conditions in a 100 μl reaction volume containing 2 units of Taq DNA polymerase (Promega Biotech) in a 1.5 mM $MgCl_2$, 0.2 mM dNTPs, reaction buffer containing 10 pmol of each primer (βAP A and βAP D, above). After incubating for 5 min. at 94°, amplification was carried out via 35 cycles of (1) 1 min. at 94°; (2) 1 min. at 65°; (3) 4 min. at 72°; followed by 10 min. at 72°; and storage at 4°.

The SOE product was then separated on a 3% agarose/TBE gel and stained with 0.5 μg/ml ethidium bromide (EtBr). The band corresponding to the SOE product was excised from the gel and DNA was isolated using a 0.45 μm spin filter, (Millipore Bedford, MA) ethanol precipitated and resuspended. An aliquot of the isolated DNA was then cut with the restriction enzyme Bam HI and cloned into the Bam HI site of the expression vector described above in Rauth *et al.*, *supra* in which the 3' cloning site had been deleted (pDT-1). The orientation of the inserted DNA was determined by restriction digests and the sequence of the inserted DNA was determined by dideoxy sequencing using a kit from U.S. Biochemicals (Cleveland, Ohio) according to the manufacturers' instructions.

The clones initially obtained contained several errors in sequence. Therefore new primers were synthesized in order to modify the sequences obtained such that a two-base pair inversion in the primer sequence βAP A was corrected and an in-frame stop codon was introduced immediately downstream of the β amyloid encoding sequences so that the C terminus of the peptide encoded would correspond to that of the naturally occurring β amyloid peptide, Glenner and Wong, *Biochem. Biophys. Res. Comm.* **120**:885-890 (1984); Masters *et al.*, *Proc. Natl. Acad. Sci.*

U.S.A. **82**:4245-4249 (1985), so that sequences deleted during the PCR amplification of the leader sequences would be restored.

The new primer sequences used to synthesize the leader sequence were:

βAP A2: 5'ATAGGATCCATGCTGCCCGGTTTGGCACT 3'; (SEQ ID NO. 5)

βAP B 5'ATTCTGCATCCGCCCGAGCCGTCCAGGCGG 3'; (SEQ ID NO. 2).

βAP B2: 5'ATTCTGCATCCGCCCGAGCCGTCCAGGCCGG 3' (SEQ ID NO. 6)

The three primers were annealed and extended under PCR conditions in a 50 μl reaction volume containing 2 units of Taq DNA polymerase (Promega Biotech) in a 1.5 mM $MgCl_2$, 0.2 mM dNTPs reaction buffer containing 10 pmol of each primer and 2 μl of heat denatured phage stock. After incubating for 2 min. at 94°, amplification was carried out in a Coy Tempcycler via 35 cycles of (1) 1 min. at 94°, (2) 1 min. at 55°; (3) 1 min. at 72°; followed by 10 min. at 72°; and storage at 4°.

Primer sequences used to amplify the β amyloid encoding sequences were:

βAP C: 5'GGCTCGGGCGGATGCAGAATTCCGACATGA 3'; (SEQ ID NO. 3); and

βAP D2: 5'ATAGGATCCTCACGCTATGACAACACCGCCCA 3' (SEQ ID NO. 7).

Sequences encoding the β amyloid peptide were amplified from one of the clones originally obtained, (psβAP1) in a 50 μl reaction volume containing 2 units of Taq DNA polymerase (Promega Biotech, Madison, Wisconsin) in a 1.5 mM $MgCl_2$, 0.2 mM dNTPs, reaction buffer containing 10 pmol of each primer, and 20 ng of template DNA from psβAP1. After incubating for 2 min. at 94°, amplification

was carried out in a Coy Tempcycler via 35 cycles of (1) 1 min. at 94°; (2) 1 min. at 55°; (1) 1 min. at 72°; followed by 10 min. at 72°; and storage at 4°.

Bands of the appropriate size (60 bp for the leader sequence and 140 bp for the β/A4 sequence) were isolated from a 3% agarose/TBE gel stained with 0.5 μg/ml EtBr, and DNA was isolated using a 0.45 μm spin filter (Millipore). The recovered DNA was then ethanol precipitated, resuspended, and then annealed and extended under PCR conditions in a 50 μl reaction volume containing 2 units of Taq DNA polymerase (Promega Biotech) in a 1.5 mM $MgCl_2$, 0.2 mM dNTPs reaction buffer containing 10 pmol each of primers βAP A2 (SEQ ID NO. 5) and βAP D2 (SEQ ID NO. 7). After incubating for 2 min. at 94°, amplification was carried out in a COY Tempcycler via 35 cycles of (1) 1 min. at 94°; (2) 1 min. at 45°, (3) 1 min. at 72°; followed by 10 min. at 72°; and storage at 4°.

A 195 bp band corresponding to the SOE product was then isolated from a 3% agarose/TBE gel stained with 0.5 μg/ml EtBr and DNA was isolated using a 0.45 μm spin filter (Millipore). The isolated DNA was cut with the restriction endonuclease Bam HI and cloned into the Bam HI site of the expression vector pDT-1 described above to generate plasmid pADAβAP+L1. The orientation of the inserted DNA was determined by restriction endonuclease digestion and the sequence of the inserted DNA was determined by dideoxy sequencing as described above.

The sequence finally obtained is given below:

5′ GGA TCC ATG CTG CCC GGT TTG GCA CTG CTC CTG CTG GCC GCC
TGG ACA GCT CGG GCG GAT GCA GAA TTC CGA CAT GAC TCA GGA
TAT GAA GTT CAT CAT CAA AAA TTG GTG TTT GCA GAA GAT GTG
GGT TCA AAC AAA GGT GCA ATC ATT GGA CTC ATG GTG GGC GGT
GTT GTC ATA GCG TGA GGA TCC 3′. (SEQ ID NO. 8)

This sequence corresponds to that encoding 17 amino acid leader and 43 amino acid β amyloid peptide portions of the human amyloid precursor protein (APP). Kang *et al., supra,* with the exception of a single base substitution in the leader sequence (shown in bold and underlined) which does not affect amino acid

composition of the peptide encoded. A map of the ADA sequence, the β/A4 sequence, and the SV40 polyadenylation sequence as present in pADAβAP+L1 is shown in Figure 1. The restriction sites shown are N=Nco I, E=Eco RI, B=Bam HI, H=Hind III. The plasmid pADAβAP+L1 also contains a T7 promoter to allow production of β/A4 antisense RNA.

Example 2

Construction of pADAhACT-1

The plasmid pADAhACT-1 was deposited with the American Type Culture Collection (Rockville, MD) on March 25, 1993 and has been assigned Accession Number ATCC 69271.

The transgene construct was prepared containing the entire coding region of the human ACT gene inserted the expression vector described by Rauth, *et al., supra.* The coding sequence of the human α1-antichymotrypsin cDNA used in the construction of plasmid pADAhACT was prepared by PCR amplification of the full length human ACT DNA insert contained in pHACT as described by Chandra, T. *et al. Biochem.* 22:5055-5061 (1983).

Primer sequences used were:

ACT-A: 5′ ATACCCGGGCAGAGTTGAGAATGGAGA 3′ (SEQ ID NO. 9) representing the 5′ end of the human ACT cDNA; and

ACT-B: 5′ ATACCCGGGTTACTGAGAGCCCCACTG 3′ (SEQ ID NO. 10) representing the 3′ end of the human ACT cDNA.

PCR was carried out in a 500 μl volume with 10 units of Taq polymerase (Promega Biotech, Madison, WI) in a 1.5 mM $MgCl_2$, 0.2 mM dNTPs in reaction buffer containing 100ng of linearized template DNA and 100 pmole each of primers ACT-A and ACT-B. Template DNA was linearized pHACT. Amplification was carried out after incubating for 2 min. at 94°, in a COY Tempcycle via 35 cycles of (1) 1 min at 94°C; (2) 1 min. at 60°C; and (3) 1 min. at 72°C; followed by 10 min. at 72°C and

storage at 4°C. Amplificati n products were analyzed by electrophoresis on 0.8% agarose gels. A 1.3 kb fragment containing the ACT coding sequence was eluted from the gel.

The ACT coding sequences obtained above were cloned into the expression vector described in Example 1 wherein the Nco I site surrounding the ATG start codon of the ADA cDNA fragment was destroyed by digestion with S1 nuclease so that the first in frame ATG codon after the major start site of transcription was the start site normally used in the human ACT gene. The ACT coding sequence was digested with Sma I to obtain blunt ends to which Bam HI linker were ligated. The Bam HI linkers were used to clone the ACT sequence containing fragment into a unique Bam HI site of the expression vector. The orientation of the insert was determined by restriction digests and the sequence of the inserted DNA was verified by dideoxy sequencing using a kit from U.S. Biochemicals according to the manufacturer's instructions. The sequence of the ACT cDNA prepared by this method is in agreement with the originally published sequence, Chandra *et al.*, *supra*, except for those differences noted previously. Hill *et al.*, *Nature* **311**:175-177 (1984); Rubin, H. *J. Biol. Chem.* **265**:1199-1207 (1990).

The plasmid pADAhACT-1 also contains a T7 promoter useful in the production of ACT antisense RNA.

Example 3

Preparation of Transgenic Animals

Both pADAhACT and pADAβAP+L1 were purified by centrifugation through CsCl gradients as described in Rauth *et al.*, *supra*. A 4.1 kb DNA fragment containing the 5′ region of the mouse adenosine deaminase gene, the human ACT cDNA and the SV40 polyadenylation sequence (ACT transgene) was excised from pADAhACT by digestion with the restriction endonucleases Nco I and Hind III. The 4.1 kb fragment was separated by electrophoresis in 0.6% low melting point agarose gel, excised from the gel and purified by phenol extraction and ethanol precipitation.

The purified 4.1 kb fragment was then injected into the male pronucleus of single cell embryos from an outbred ICR strain mouse originally of Swiss Webster background (Charles River Laboratories Boston, MA and Jackson Laboratory, Bar Harbor, Maine and National Cancer Institute Fredrickson, Maryland) using the method described by Ross, *et al., Proc. Nat'l. Acad. Sci. USA* 82:5880-5884 (1985). Injected embryos were then implanted into pseudopregnant females as described in Ross *et al., supra* and allowed to proceed to term.

Similarly, a 2.9 kb fragment containing the 5′ region of the mouse adenosine deaminase gene and the APP leader sequence fused to the 5′ end of the β/A4 coding sequence and the SV40 polyadenylation sequence (β/A4 transgene) was excised from the plasmid pADAβAP+L1 using Nco I and Hind III. The fragment was purified as described above and single cell mouse embryos were injected with the purified 2.9 kb transgene.

Example 4

Detection of Transgene DNA in Transgenic Animals

Genomic DNA was prepared from tail tissue of potential founder mice produced by the method of Example 3 according to the method described in Rauth *et al., supra.* DNA was then analyzed for the presence of the ACT transgene or the β/A4 transgene by Southern hybridization. Genomic DNA (20 μg) was digested to completion with Eco RI and was analyzed by electrophoresis on 0.8% agarose gels. Gels were blotted and the blots were hybridized with ^{32}P-labeled probes corresponding to the SV40 polyadenylation sequences. SV40 sequences were used as probes to eliminate the possibility that coding sequence probes might cross-react with endogenous ACT or β/A4 coding sequences. Blots were then washed at 68°C for 30 minutes in 2x SSC twice, at 68°C for 30 minutes in 1x SSC twice and finally for 30 minutes in 0.5x SSC. Blots were then exposed to x-ray film at -70°C before developing.

Of 28 potential founder mice, five f under mice were identified by virtue of the presence of a 4.1 kb band n Southern blots corresponding to ACT transgene DNA. Founder mice were bred to non-transgenic ICR mice and three of the founders (strain numbers 5, 6, and 19) showed transmission of the transgene to their offspring. Figure 3 shows Southern blot analysis DNA from one-month old offspring from strain #19 (lanes 1 and 2), strain #5 (lanes 3-6), and strain #6 (lanes 7-11) and reveals a band of about 4.1 kb which hybridized to the SV40 polyadenylation sequence probe. Since the ACT construct contains a single Eco RI (see Figure 2) site, the presence of 4.1 kb band indicates that the ACT construct was integrated into the mouse genome as tandem repeats. Lane 12 contains genomic DNA derived from tail tissue of a non-transgenic mouse and does not show the presence of DNA corresponding to the transgene.

Similarly, Southern blot analysis of DNA from tail DNA of potential founder mice containing the β/A4 transgene identified 2 potential founder mice. Southern blot analysis of Eco RI digested DNA from the offspring of these founders (Figure 4 lanes 2 and 8) revealed transmission of transgene by virtue of the presence of bands hybridizing with the SV40 polyadenylation sequence probe.

Example 5

Expression of the ACT Transgene In Mouse Brain

Expression of the ACT transgene in the brains of each strain of transgenic mice were analyzed by a reverse transcriptase - polymerase chain (RT-PCR) reaction.

Transgenic animals carrying the ACT transgene and wild-type animals were sacrificed at 2 months of age, and total brain RNA was prepared as described in Davis *et al.* eds. *Basic Methods in Molecular Biology*, Elsevier pp. 130-135 (1986).

RT-PCR was performed as described by Kawasaki, E.S. *Amplification of RNA*, in *PCR Protocols, A Guide to Methods and Applications*. Innis, M.A. *et al.*

eds., *Academic Press Inc.*, pp. 21-28 (1990). RT-PCR was carried out by reverse transcribing 1 μg of total brain RNA using 200 units of Moloney Murine Leukemia Virus reverse transcriptase (Gibco-BRL, Gaithersberg Maryland) and 100 pmoles of random hexamers (primers) (Pharmacia-LKB, Piscataway NJ) in a total reaction volume of 20 μl according to the manufacturer's instruction. Transgene specific primers were chosen so that a 340 base pair fragment from the 3′ end of the ACT transcript would be amplified.

The primer sequences used are as follows:

rPCRhACT1-5′: 5′ CACCAGCAAGGCTGACCTGT (SEQ ID NO. 11);

rPCRhACT1-3′: 5′ GTTGTGGTTTGTCCAAACTC (SEQ ID NO. 12).

PCR amplification was carried out in a 50 μl reaction volume using 2 units of Taq Polymerase (Promega Biotech) in 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 4 μl of the reverse transcription reaction and 10 pmoles of each transgene specific primer. All reaction components were assembled and, after a 2 min. incubation at 94°C, amplification was carried out in a COY Thermocycle at the following settings: 1 min. at 94°C; (2) 1 min. at 60°C; and (3) 1 min. at 72°C; followed by 10 min. at 72°C and storage at 4°C. Half of each reaction was then analyzed by electrophoresis on a 2% agarose gel and staining with ethidium bromide (0.5 μg/ml).

Figure 5 shows the results of the RT-PCR analysis. RNA derived from the brains of transgenic mouse strain #19 (Lanes 1 and 2), strain #5 (lanes 3 and 4), strain #6 (lanes 6 and 7), and wild-type mice lacking the ACT transgene (lanes 8 and 9) were analyzed as described above. Lanes 1, 3, 5 and 9 represent reactions which were reverse transcribed prior to amplification, and lanes 2, 4, 5 and 9 represent control reactions which were performed without reverse transcription prior to amplification. Additional positive and negative amplification controls consist of amplification reactions performed using either tail DNA derived from one of the transgenic mice (lane 9) or in the absence of any nucleic acid template (lane 10). The

expected PCR product generated if transgene specific RNA was present was 340 bp in size.

As illustrated in Figure 5, all three strains tested for expression of the ACT transgene specific RNA showed expression in the brain as revealed by the presence of a 340 bp band (lanes 1, 3, 5) on agarose gel electrophoresis which was also seen when DNA from a transgenic mouse containing the ACT transgene was used as a template for PCR (lane 9).

Example 6

In situ Hybridization Analysis of ACT Transgene Expression in Transgenic Mouse Brain

In order to localize expression of ACT specific messenger RNA in transgenic mouse brain, tissue was analyzed using *in situ* hybridization. Brains from wild-type and transgenic mice were harvested between 5 and 10 months of age and were immersed in O.C.T. (Miles, Inc. Diagnostics Division, Elkhart, IN) embedding compound, frozen in powdered dry ice, and stored at -80°C prior to sectioning.

Frozen, embedded brain tissue was cut into 8 μm sections on a cryostat and sections were thaw-mounted on poly-L-lysine coated slides. Mounted sections were then fixed in 4% paraformaldehyde and blocked with 0.5 ml acetic anhydride diluted in 200 mls 0.1M triethanolamine pH 8.0 prior to hybridization. (Current Protocols in Molecular Biology, Ausubel *et al.*, eds. Greene Publishing Associates, Brooklyn, NY, 1991). ^{35}S-labeled antisense RNA probe was transcribed *in vitro* from linearized pADAhACT using T7 RNA polymerase (Promega Biotech). The antisense probe used corresponds to a 590 bp fragment from the 5' end of the human ACT gene. Hybridization was carried out in hybridization buffer containing 50% formamide, 10 mM dithiothreitol (DTT), 2 x SSC, 1 mg/ml yeast tRNA, 1 mg/ml bovine serum albumin (BSA), 1 mg/ml sonicated salmon sperm DNA. Hybridization was carried out on a slide warmer at 55°C 1 mg/ml with 15 μl hybridization buffer per slide under glass cover slips (Corning, N.Y.) sealed with rubber cement to

prevent evaporation. After hybridization, slid s were rinsed in 50% formamide/12 x SSC until cover slips fell off, followed by a 15-minute was in 2 x SSC (room temperature), 30 minutes in 10 μg/ml RNAase A/0.5 M NaCl, 1 mM EDTA (37°C); 30 minutes in RNAase A buffer (0.5 M NaCl; 1 mM EDTA (37°C), 30 minutes in 2 x SSC, 10 mM β-mercaptoethanol (βME) (55°C); 30 minutes in 1 x SSC, 10 mM βME (55°C), 30′ in 0.5 x SSC; 10 mM βME (55°C), 30′ in 0.1 x SSC; 10 mM βME (55°C) then dipped in Kodak NTB2 Liquid Nuclear Tract Emulsion (Eastman Kodak, Rochester, New York) and exposed for one month at 4°C in a light-tight desiccated chamber. After exposure, slides were photographically developed and the sections were counterstained with hematoxylin and eosin (Figure 6A and B) or cresyl violet (Figure 6C and D).

Figure 6 shows the results of *in situ* hybridization analyses. High level expression of the ACT transgene has seen throughout the CNS (by virtue of hybridization of the ACT specific antisense probes shown by the presence of silver grains) including cortex (C), hippocampus, dentate gyrus (O), basal ganglia, thalamus (T), hypothalamus (H), and cerebellum (Cb). Figure 6, panel A represents a coronal section from a transgenic mouse (strain 5) illustrating ACT expression in the hippocampus and surrounding cortex. Figure 6, panel B shows a coronal section from a wild-type mouse hybridized with the same probe which shows no hybridization. Figure 6, panel C represents a sagittal section from a transgenic mouse (strain 5) showing ACT expression throughout the brain. Figure 6, panel D represents a sagittal section from a wild-type mouse hybridized to the same ACT transgene which shows no hybridization. These studies show that most, if not all, regions of the CNS thought to be important in the development of Alzheimer's disease pathology showed significant expression of the ACT transgene. Hybridization was observed throughout the grey matter regions of the brain indicating that expression was predominantly neuronal, while white matter regions (e.g. corpus callosum) showed little or no hybridization. The distribution of ACT mRNA expression closely mimics the general distribution of neurons in all of the brain regions studied. In particular, the granule cell layer of the hippocampus and the

dentate gyrus were the most intensely labeled. Layer 1 of the cerebral cortex, which contains relatively few neurons had the lowest hybridization signal in the cortex, while other more neuron-rich regions showed more intense labeling. These observations suggest that the ACT transgene expression pattern in these animals was predominantly neuronal, with some expression occurring in other cell types.

Example 7

Histologic Examination of Brains from Transgenic Animals

The brains of human patients with Alzheimer's disease show characteristic lesions including diffuse amyloid plaques, neurofibrillary tangles, senile neuritic plaques and others. To determine if expression of the ACT transgene in transgenic mice correlated with the appearance of any of the lesions described above, eight-month-old transgenic offspring from each of the three strains (5, 6, and 19), as well as control mice of varying ages were analyzed using two different methods.

Following an intraperitoneal overdose of Nembutal sodium, brains were prepared for analysis by perfusing mice systemically with 10% formalin. Brains were removed and post-fixed in 40% formalin for 3-7 days at 4°C, and cryoprotected by immersion in 30% sucrose until tissue sank to the bottom of the container. Brains were then frozen on powdered dry ice and sectioned at 50 μm intervals on a cryostat. Series of parasagittal sections were impregnated with silver according to the method of Campbell *et al.*, *Soc. Neurosci. Abst.* **13**:678 (1987) which has previously been shown to be both highly sensitive and specific in the staining of the β/A4 amyloid lesions found in the brain of AD patients. Braak, *et al.*, *Brain Pathol.* **1**:213-216 (1991). Figure 7 shows photomicrographs of parasagittal sections of the brain of a transgenic mouse expressing the ACT transgene (panels A-E), and an age-matched non-transgenic ICR control mouse (panel F). Plaque-like lesions morphologically indistinguishable from the diffuse amyloid plaques of AD patients are seen in virtually every region of the transgenic mouse brain including the hippocampus (arrowheads in Panel A, C and E), cerebral neocortex (arrows in Panel D), midbrain (arrowheads

in Panel B), basal telencephalic nuclei (a.k.a basal ganglia) diencephalon, cerebellum, pons, medulla and spinal cord. Panel E shows a higher power view of the hippocampal region from the section shown in Panel C and demonstrates the plaque-like lesions in the hippocampus (arrows) and adjacent neocortex. Panel F illustrates the absence of such lesions in age matched non-transgenic ICR mouse brain. All the brains from transgenic offspring exhibited widespread argyrophilic lesions from 10 to about 250 μm in diameter (Figure 7). These plaque-like lesions were morphologically indistinguishable from the diffuse amyloid plaques found in the brain of AD patients. Tagliavini, *et al., Neuropathol. Exptl. Neurol* **47**:332; Yamaguchi, *et al., Acta Neuropathol.* **76**:541-544 (1988); Joachim, *et al., Am. J. Pathol.* **135**:309-319 (1989); Bugiani, *et al., Neurosci. Lett.* **103**:263-268 (1989); and Ikeda *et al., Acta Neuropathol.* **78**:137-142 (1989). The lesions were situated in virtually every region of the mouse brains, including the hippocampus, diencephalon, cerebral neocortex, basal telencephalic nuclei (a.k.a. "basal ganglia"), midbrain, cerebellum, pons, medulla and spinal cord.

Example 8

Immunohistochemical Analysis for ACT in Plaques

One of the most common features of the plaques found in the brains of AD patients is the presence of ACT. Abraham, *et al., Cell* **52**:487-501 (1988); and Abraham, *et al., Neurobiol. Aging* **11**:123-129 (1990). Antibodies against ACT label both the amyloid cores of neuritic plaques, Abraham *et al., supra,* well as the fine amorphous cerebral amyloid deposits as described by Shoji, *et al.* in *Alzheimer's disease and related disorders*; Iqbal, *et al.* eds. pp 1049-1059, New York, Alan R. Liss Inc. (1989).

In order to test for the presence of ACT in the brains of transgenic animals, animals were sacrificed and fixed in formalin as described in Example 7. Brains were then frozen and series of 50 μm thick parasagittal sections were obtained on a cryostat. Brain sections were incubated in 1:500 - 1:2000 dilutions of rabbit

anti-α1-antichymotrypsin in phosphate buffered saline - sodium azide. (Zymed Laboratories, Inc. So. San Francisco, CA 94080). Brains were then processed according to the avidin-biotin-peroxidase method described by Kuljis, R. *et al.*, *Neurosci. Lett.* 106:49-54 (1989). Human tissues showing histologically confirmed AD were obtained at the time of autopsy and were processed for labeling for ACT following the same method used for mice.

Figure 8 illustrates the results of this analysis. Figure 8, panels A-D illustrate the presence of immunolabeled ACT containing plaques in the brains of transgenic mice. The brains of human patients with AD also show the presence of immunolabeled ACT (Panels E and F). Like their human counterparts, sections from transgenic mice stained with rabbit antihuman anti-α1-antichymotrypsin displayed plaque-like or cloud-like aggregations of ACT immunoreactive material within the plaques. These aggregations appeared in the parenchyma proper (Panels A and B), in the subpial region (from the submolecular layer of the cerebellum) (Panel C) and in the perivascular spaces (Panel D). No obvious qualitative or quantitative feature distinguished the plaques seen in the transgenic mice from those seen in human tissue from confirmed AD patients even when differential interference contrast optics were used. These results demonstrate that expression of the ACT transgene in the brain of transgenic animals results in the production of plaques that are morphologically identical to the diffuse amyloid plaques seen in human patients with AD whether silver staining or immunostaining was used.

Example 9

Immunohistochemical Analysis for the β/A4 Peptide

Additional studies were conducted to determine whether the plaque-like lesions described in Example 7 contained the APP-derived amyloid protein. Series of sections adjacent to those taken in Example 7 were immunolabeled with a panel of antibodies including a polyclonal antiserum raised against the β/A4 fragment of the human amyloid protein as described by Masters C.L. *et al.*, *EMBO J.* 41:2257-2763

(1985) (the "Masters antiserum"), a polyclonal antiserum raised against synthetic β-amyloid (Boehringer Mannheim, Indianapolis, Indiana Cat. No. 1 381 431) and a monoclonal antibody (MAb 22C11) raised against purified recombinant human APP as described by Weidman, A. *et al.*, *Cell* 57:115 (1985) (Boehringer Mannheim Cat. No. 1 285 262). These three immunological probes were previously shown to label the amyloid component of plaques in patients with AD, but not native cell membrane-associated APP. Masters *supra,* Weidman *supra.*

Mice were fixed and prepared for histology as described above and tissues were treated with antibodies in dilutions of 1:500-1:2000 after pretreatment of the tissue with 70% formic acid. Tissues were then stained using the avidin-biotin-peroxidase method as described by Kuljis *et al.*, *supra.* The best results were obtained in material intensified according to the protocol of Gallyas *et al.*, *J. Histochem. and Cytochem.* 30:183, (1982) after immunolabeling. Tissue from transgenic and age-matched control mice and from patients with confirmed AD was processed under identical conditions.

Both the antisera and the monoclonal antibody used in these studies recognized lesions similar to those revealed by the method of Campbell *et al.*, *supra* as (see Example 7). Figure 9A-C illustrate β/A4 immunopositive deposits in transgenic mouse brain immunolabeled with the Masters antiserum. Figure 9A depicts lesions in the interface between the pontine tegmentum and base. Figure 9B depicts lesions in the dorsal pontine tegmentum. Figure 9C depicts lesions in the molecular layers of the cerebellum. In figure 9C, PCL denotes the Purkinje cell layer and the arrows delineate the borders of a large β/A4 deposit. Most plaques were 8-55 μm in diameter and displayed irregular contours. They contained a variable admixture of 8-12 μM clumps, puncta, and a hazy amorphous background that clearly distinguished them from surrounding unlabeled tissue. These lesions were situated in the same regions in which agyrophilic plaques were seen in adjacent sections as described in Example 7. No plaque-like lesions or deposits were observed with these methods in any of the five control non-transgenic ICR mice (ranging in age from 4-17 months). Furthermore, control mice did not show amyloid type deposits of any type,

including the type seen occurring spontaneously in C57/BL6 as reported by Jucker *et al., supra.* Thus, the amyloid-containing plaques observed in the transgenic animals described herein are attributable to the presence of the ACT transgene.

Example 10

Testing of Therapeutic Interventions for Alzheimer's Disease

The transgenic animals of the present invention may be used to test potential therapeutic interventions for the treatment and prevention of AD disease. Potential interventions range from but are not limited to traditional types of pharmacologic intervention (e.g., drugs) to molecular therapeutic interventions including the utilization of antisense RNA, ribozymes, tagged-triplex DNA/RNA molecules, small peptides derived from dominant negative mutations, peptide nucleic acid molecules (PNA), peptides interfere with the biochemical process of plaque deposition, antibodies directed to plaque-producing proteins and others, all of which are well known in the art. These treatments may be administered after the onset of plaque-formation or may be used in a prophylactic capacity.

To ascertain the effects of potential therapeutic interventions, animals may be sacrificed at various times after intervention and their brains examined for the presence of diffuse amyloid plaques, neurofibrillary tangles and other histologic signs of Alzheimer's disease using the standard histologic and immunohistologic techniques described above, which are widely known in the art. The brains from these animals may also be examined for the presence of α1-antichymotrypsin-specific or β/A4-specific mRNAs using RT-PCR, Northern blotting RNase protection assays or by *in situ* hybridization. Those therapeutic interventions showing an effect on the expression of transgenes and/or on the process of plaque formation would then merit more intensive investigation.

Example 11
Other Transgenic Animal Model Systems For Alzheimer's Disease

The present invention is also directed to transgenic animal model systems comprising non-human mammals other than mice. Such animal models may differ from mice in their natural aging processes and rate of aging and will allow a more detailed investigation of the role of aging in the onset of plaque formation in transgenic animals.

Transgenic animals including transgenic rabbits, transgenic pigs, transgenic rats, and other transgenic animals have been described. (*See for example*, Hammer, *et al.*, *Nature* 315:680-683 [1985]). Transgene constructs useful in other transgenic animal model systems for AD would include regulatory sequences capable of directing expression of a coding sequence in the neurons of transgenic animals as well as other sequences such as leader sequences which may be important to protein secretion process. The coding sequences useful in such constructs include the coding sequence for ACT, β/A4 peptide encoding sequences and other coding sequences whose expression can result in the development of the characteristic histopathologic signs of AD.

Example 12
Transgenic Animal Test Systems for Neurologic Deficit

In humans, there appears to be a correlation between the presence of the characteristic histopathology of AD and the neurologic deficits associated with the disease. These deficits include memory loss and other cognitive disorders. These possible relationships may also be studied in more detail using the transgenic animals of the present invention.

For example, animals of the present invention may be taught certain tasks such as the Morris water maze task which measures spatial learning in animals.

As the disease progresses in the animal as evidenced by the increasing abundance of plaques and ther pathophysiologic features of AD, the animals may be retested for their ability to perform the same tasks or for their ability to learn new tasks. Other methods for testing learning (e.g. avoidance testing) are within the skill of the ordinary person in the art of animal psychology, pharmacology, neurology and physiology.

In addition, such tests will be useful in assessing whether or not therapeutic innervations such as those described in Example 10 have any effect on the neurologic deficits which may arise in the transgenic animals of the present invention.

Example 13

Environmental Causes of AD

Proposed environmental causes of AD include head trauma, viral infection, aluminum toxicity and others. Gautrin *et al.*, *Can. J. Neurol. Sci.* 16:375-387 (1989). One common aspect among these otherwise highly diverse environmental factors is their potential for causing localized inflammation within the brain. A major feature of the inflammatory response is the rapidly increased production of a set of "acute phase response" proteins among which ACT is one of the earliest and most prominent. Travis, *et al.*, *Ann. Rev. Biochem.* 52:655-709 (1983). Other acute phase response proteins which have been postulated to play a role in the formation of amyloid in the brain are the cytokines IL-1/IL-6. Vandenabeele *et al.*, *Immunol. Today* 12:217-219 (1991).

Many viruses including herpes simplex viruses, varicella zoster, measles virus, entero-viruses and others are known to cause acute infections of the CNS. Gautrin *et al.*, *supra.* Infection with these agents can lead to the kinds of localized inflammatory reactions that could lead to elevated ACT expression and possibly to the formation of diffuse plaques, neurofibrillary tangles and other characteristic histopathology of AD.

The transgenic animals of the present invention may be used to test whether or not the aforementioned environmental insults to the brain can in fact influence the rate of formation or the extent of AD-like pathology. For example, animals of the present invention may be infected with viruses suspected of playing a role in the etiology of AD after which expression of the transgene or plaque formation may be monitored.

Similarly, animals of the present invention may be used to investigate the effects of head trauma, and environmental toxins including metals and organic chemicals, on the progression of the neurologic and pathologic signs of AD.

Example 14

Cell Culture Systems for the Study of Alzheimer's Disease

The present invention is also directed to cell culture systems which express genes which play a role in the development of AD.

Cell cultures derived from the transgenic animals may be used to test agents which may influence the expression of genes which play a role in the development of AD. Cell cultures derived from the animals of the present invention may also be used as test systems for agents or interventions which may alter the translation of mRNAs such as ACT mRNA or β/A4 mRNA or which may alter the post-translational modification of proteins such as β/A4 peptide or ACT. The preparation of cell cultures from animals can be accomplished using standard techniques known to those of ordinary skill in the art of cell culture.

Example 15

Transfection of PC-12 Cells with pADAhACT

Cell lines useful in the study of AD may also be prepared by transfecting cells in culture with transgenes of the present invention. Transfection may be accomplished using well-known techniques such as electroporation, calcium

phosphate co-precipitation, retrovirus vector mediated gene transfer, microinjection or other techniques known in the art.

Transfection may be carried out by cotransfecting the transgene of interest with a selectable marker such as the bacterial neo^r marker which confers resistance to the antibiotic G418 on cells expressing the neo^r. Southern, *et al.* J. Mol. Appl. Genet. 1:327-341 (1982). As is well known in the art, a significant percentage of the cells expressing the neo^r gene will also express the cotransfected transgene. Thus, transformed cells surviving selection in G418 may then be assayed for expression of the transgene by any of a wide variety of techniques known to those of ordinary skill in the art.

Transfected cells may also be prepared as described above without cotransfection with a selectable marker. In that case cells may be transfected with a transgene and assayed for expression of the transgene from about 24 to about 72 hours after transfection without any intervening selection.

In order to determine whether or not pADAhACT could be expressed in cells in culture and across species, pADAhACT was transfected into rat cells in culture. More specifically, subconfluent monolayers of rat pheochromocytoma cells (PC-12 cells) were cotransfected with the plasmid pADAhACT and a pTZ19U based plasmid containing (in 5' to 3' orientation) the minimal ADA promoter (Rauth *et al. supra*) and the first 40 amino acids coded for by the ADA cDNA (Yeung, *et al., J. Biol. Chem.* **260**:10299-10307 [1985]), linked to the entire neo^r coding sequence followed by an SV40 polyadenylation signal.(Southern, *et al.* J. Mol. Appl. Genet. 1:327-341 (1982). The plasmids were co-transfected using a calcium phosphate-based transfection method as described in *Current Protocols In Molecular Biology*, Ausubel *et al.*, eds. *supra* using a HEPES-buffered solution to form the calcium phosphate/DNA co-precipitate. After transfection, cells were incubated for 48 hrs. before selection in G418. Transfected cells were allowed to grow for two weeks in G418 before G418 resistant colonies were picked and expanded to assay for expression of the ACT transgene.

To assay for the expression of the ACT transgene, RNA was prepared as described in Ausubel, *supra* and was assayed for the presence of ACT-specific RNA by RT-PCR as described in Example 5. Results of this analysis revealed that ACT-specific RNA was present in cells transfected with pADAhACT by virtue of the presence of a 340 bp band on an agarose gel, while no ACT-specific RNA was detected in control non-transfected cells.

While the invention has been described in terms of non-limiting preferred embodiments, it is intended that the present invention encompass all modifications and variations within the scope of the appended claims which may occur to those skilled in the art.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: University of Illinois
(B) STREET: 352 Administration Building, 506 S. Wright Street
(C) CITY: Urbana
(D) STATE: Illinois
(E) COUNTRY: United States of America
(F) POSTAL CODE: 61801

(ii) TITLE OF INVENTION: Transgenic Animal Models for Alzheimer's Disease

(iii) NUMBER OF SEQUENCES: 12

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Marshall, O'Toole, Gerstein, Murray & Borun
(B) STREET: 6300 Sears Tower - 233 South Wacker Drive
(C) CITY: Chicago
(D) STATE: Illinois
(E) COUNTRY: USA
(F) ZIP: 60606-6402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Clough, Ph.D, David W.
(B) REGISTRATION NUMBER: 36,107
(C) REFERENCE/DOCKET NUMBER: 31188

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 312/474-6300
(B) TELEFAX: 312/474-0448

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
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(B) TYPE: nucleic acid
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(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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(B) TYPE: nucleic acid
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(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human α1-antichymotrypsin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human α1-antichymotrypsin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTTGTGGTTT GTCCAAACTC 20

We Claim:

1. A transgenic non-human mammal all of whose germ cells and somatic cells contain a transgene introduced into said mammal or an ancestor of said mammal, said transgene comprising regulatory sequences and a coding sequence, said regulatory sequences being capable of directing predominantly neuronal expression of said coding sequence in brain and wherein said coding sequence is selected from the group consisting of sequences coding for β/A4 peptide and α1-antichymotrypsin.

2. The mammal of claim 1 wherein said regulatory sequences comprise a 2.7 kb upstream fragment of the murine adenosine deaminase gene.

3.. The mammal of claim 1 and 2 wherein said coding sequence comprises the coding sequence for the β/A4 peptide.

4. The mammal of claim 3 wherein said coding sequence further comprises a leader sequence, said leader sequence consisting essentially of the leader sequence of an amyloid precursor protein gene.

5. The mammal of claims 1 and 2 wherein said coding sequence is the coding sequence for human α1-antichymotrypsin.

6. An autonomously replicating plasmid comprising regulatory sequences and a coding sequence, said regulatory sequences being capable of directing neuronal expression of said coding sequence and wherein said coding sequence comprises a sequence coding for the β/A4 peptide.

7. The plasmid of claim 6 wherein said regulatory sequences comprise a 2.7 kb upstream fragment of the murine adenosine deaminase gene.

8. The plasmid of claim 6 further comprising a leader sequence, said leader sequence consisting essentially of the leader sequence of an amyloid precursor protein gene.

9. An autonomously replicating plasmid comprising regulatory sequences and a coding sequence, said regulatory sequences being capable of directing neuronal expression of said coding sequence and wherein said coding sequence comprises a sequence coding for α1-antichymotrypsin.

10. The plasmid of claim 9 wherein said regulatory sequences comprise a 2.7 kb upstream fragment of the murine adenosine deaminase gene.

11. A cell line derived from the mammal of claim 1.

12. A cell line transfected with the transgene according to claim 4.

13. A cell line transfected with the transgene according to claim 5.

14. The mammal of claim 1, said mammal being a rodent.

15. The mammal of claim 14, said mammal being a mouse.

16. An autonomously replicating plasmid comprising a regulatory sequence and a coding sequence, said regulatory sequence being capable of directing neuronal expression of said coding sequence and wherein said coding sequence further comprises a leader sequence.

17. The plasmid of claim 16 wherein said regulatory sequence comprise a 2.7 kb upstream fragment of the murine adenosine deaminase gene.

18. The plasmid of claim 17 wherein said leader sequence comprises the amyloid precursor protein leader sequence.

19. The plasmid of claim 17 wherein said leader sequence comprises the α1-antichymotrypsin leader sequence.

FIGURE 1

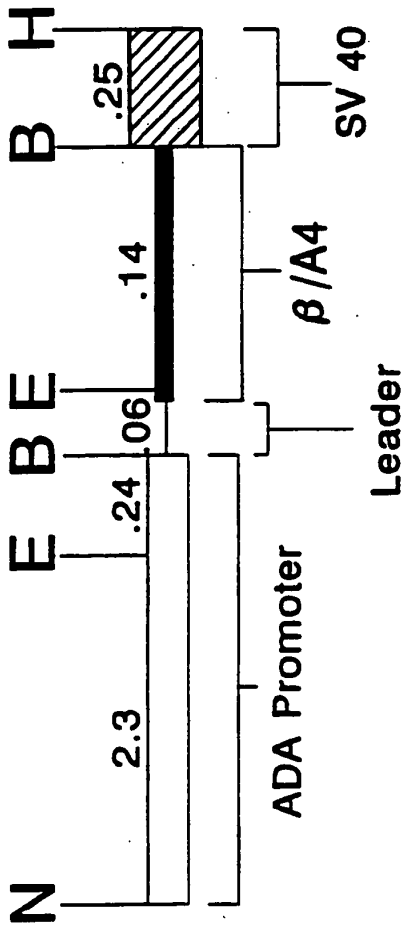
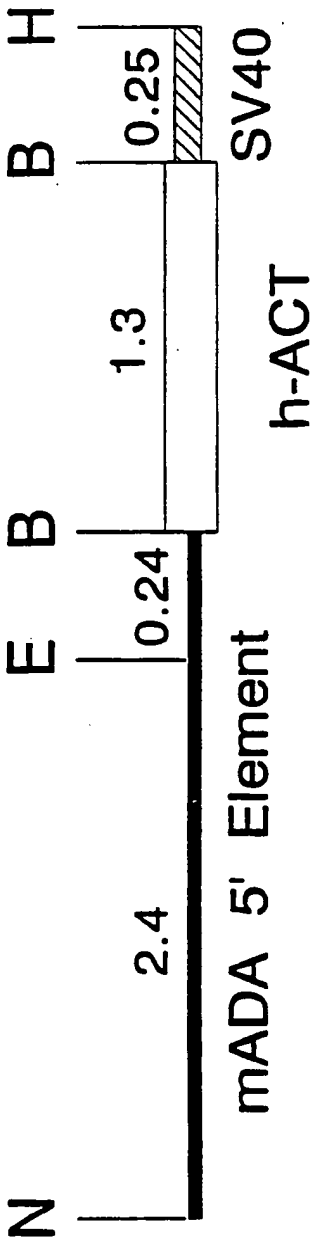


FIGURE 2



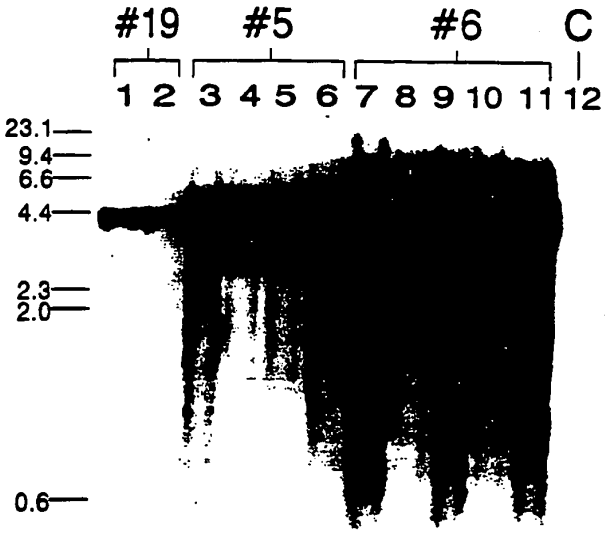
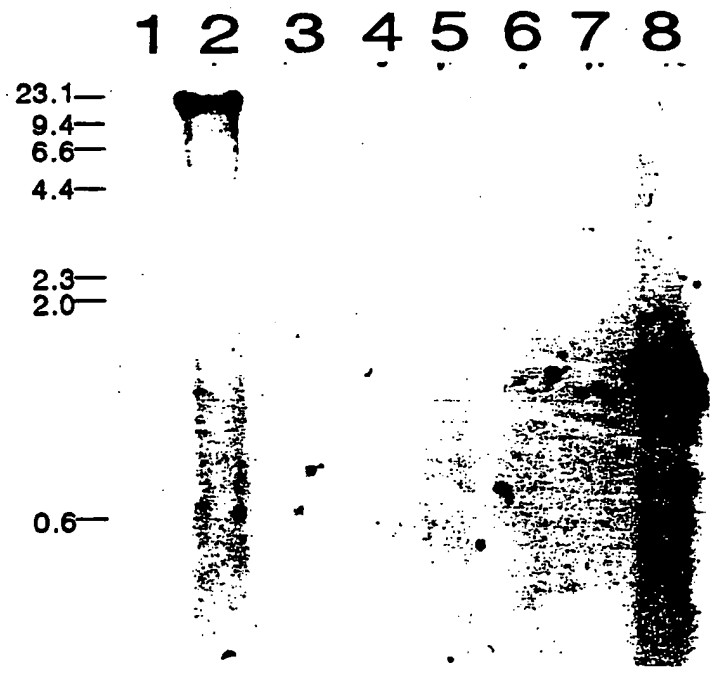


FIGURE 3

SUBSTITUTE SHEET (RULE 26)



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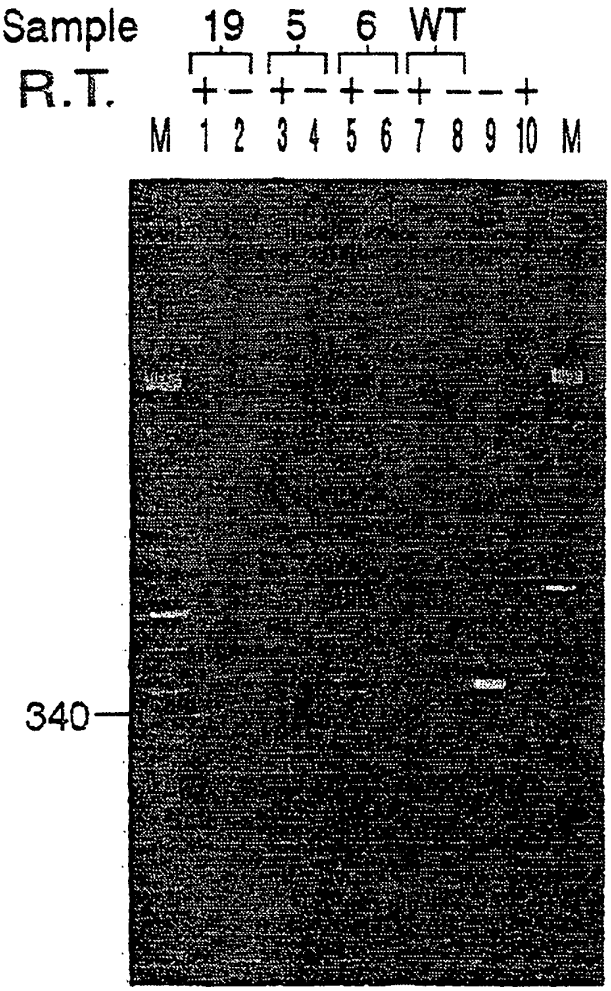

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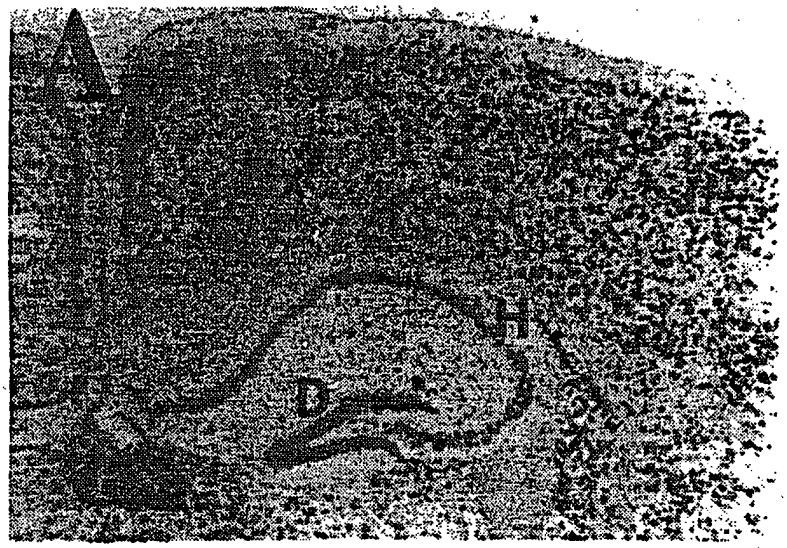

FIGURE 6A

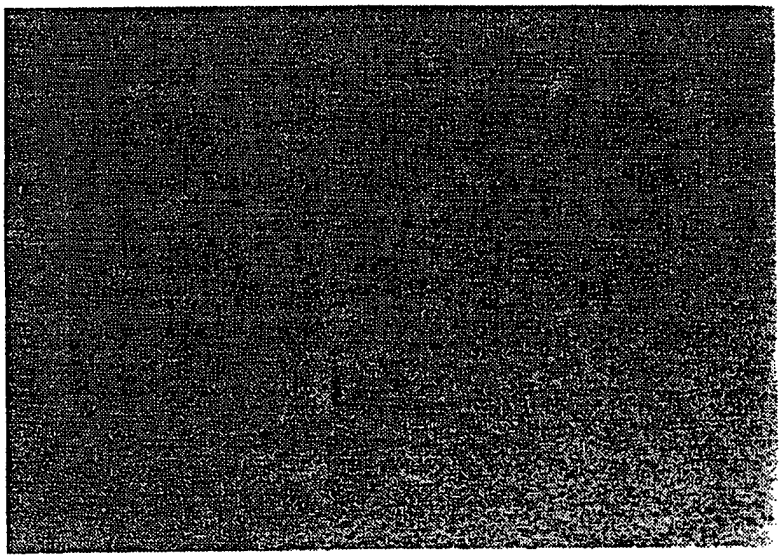

FIGURE 6B

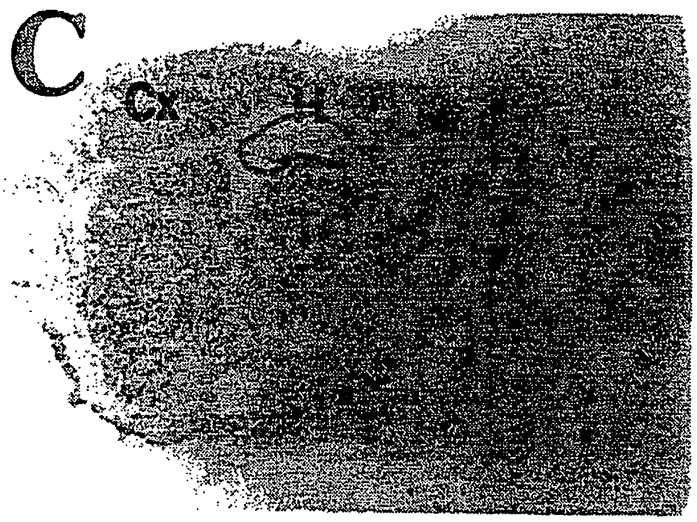

FIGURE 6C

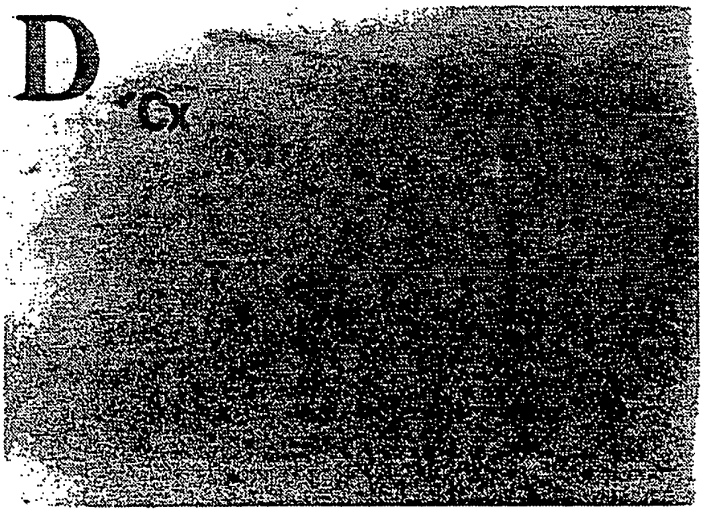

FIGURE 6D

FIGURE 7A

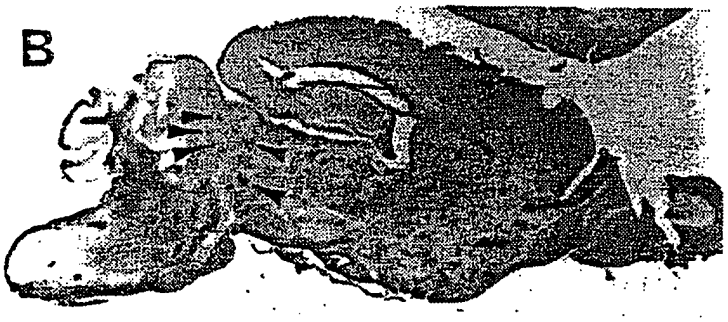

FIGURE 7B

FIGURE 7C

FIGURE 7D

FIGURE 7E

FIGURE 7F

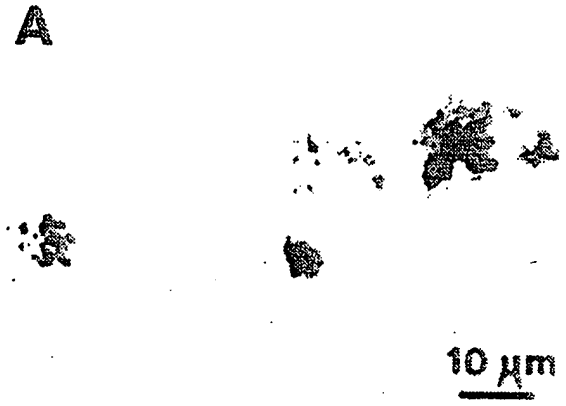

FIGURE 8A

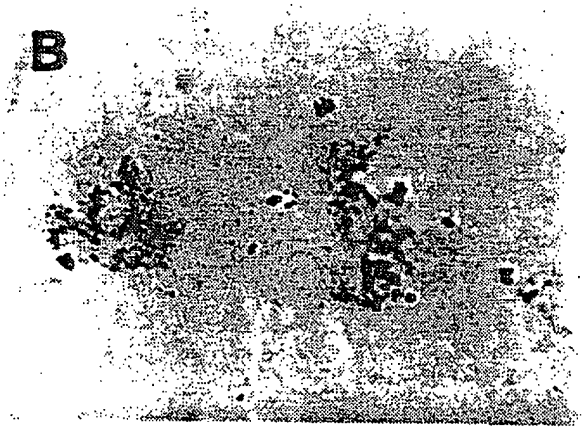

FIGURE 8B

C

FIGURE 8C

D

FIGURE 8D

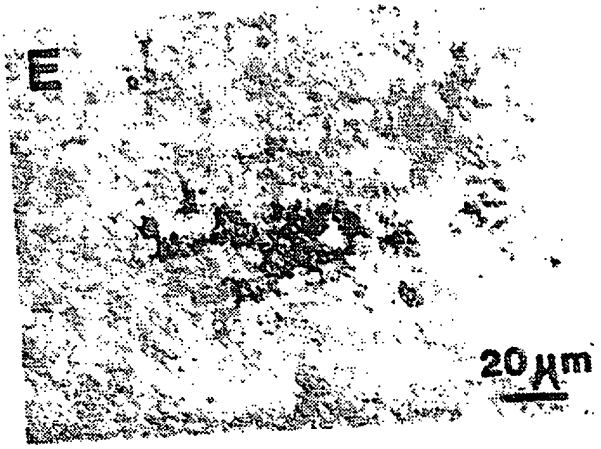

FIGURE 8E

FIGURE 8F

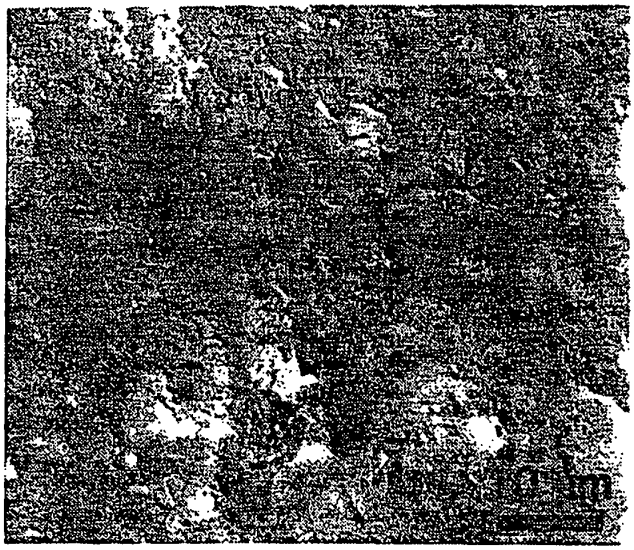

FIGURE 9A

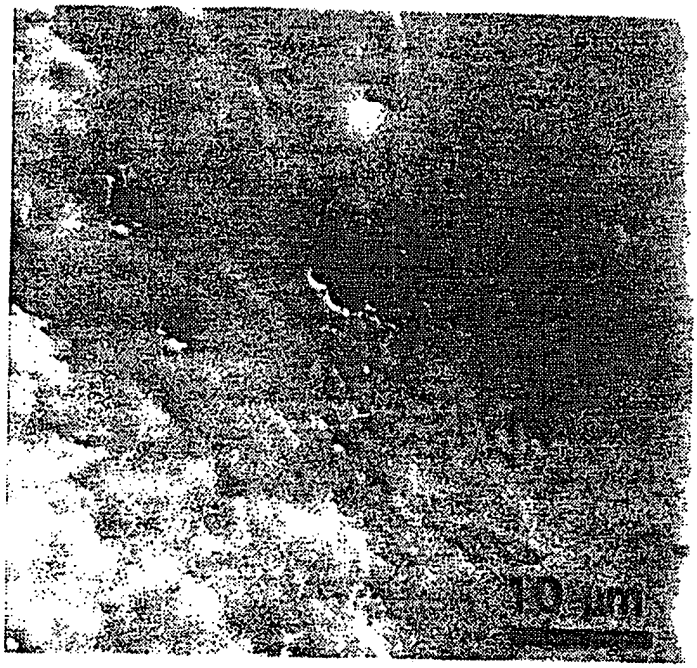

FIGURE 9B

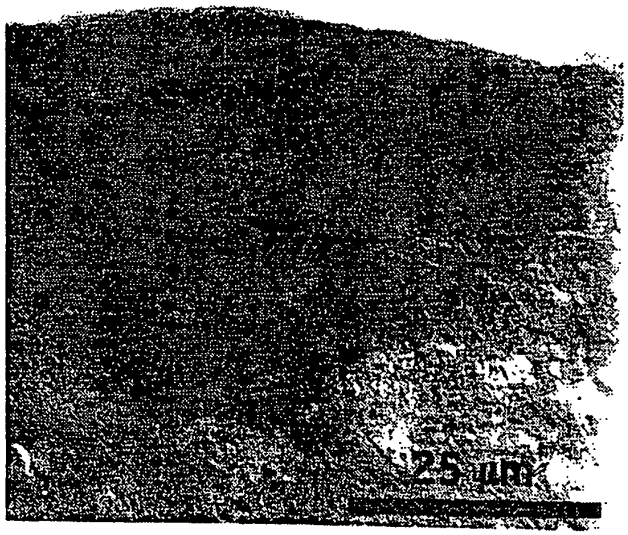

FIGURE 9C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04026

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 5/00
US CL :800/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Volume 352, issued 18 July 1991, D. Quon et al, "Formation of β-Amyloid Protein Deposits in Brains of Transgenic Mice", pages 239-241, see entire document.	1-2, 11 and 14-15
Y	Nature, Volume 341, issued 12 October 1989, Nostrand et al, "Protease Nexin-II, a Potent Antichymotrypsin, Shows Identity to Amyloid β-Protein Precursor", pages 546-549, see entire document.	1-2, 5, 11 and 14-15
Y	Biochemistry, Volume 22, No. 22, issued 25 October 1983, T. Chandra et al, "Sequence Homology Between Human α1-Antichymotrypsin, α1-Antitrypsin and Antithrombin III", pages 5055-5060, see page 5058, figure 4.	1-2, 5, 11 and 14-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 JULY 1994	27 JUL 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Deborah Crouch Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04026

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1,2,5,11,14 and 15

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04026

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claim(s)1,2,5,11,14 and 15, drawn to a transgenic mammal which contains a transgene for alpha1-antichymotrypsin and a cell line derived from the mammal, classified in Class 800, subclass 2.

Group II, claim(s) 1-4,11,14 and 15, drawn to a transgenic mammal which contains a transgene for beta/A4 peptide and a cell line derived from the mammal, classified in Class 800, subclass 2.

Group III, claim(s) 6-10,12-13 and 16-19, drawn to a plasmid containing the DNA sequence encoding alpha1-chymotrypsin or the beta/A4 peptide and cell lines transfected with the plasmid, classified in Class 435, subclass 320.1.

The inventions of group I and group II are mutually exclusive and independent as they are drawn to transgenic mammals with separate and distinct DNA sequences for the transgene. The mammals of group I are not required for the mammals of group II, and vice versa. The invention of groups I and II, and group III are mutually exclusive and independent as the plasmid can be used to produce proteins *in vitro*. These inventions are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.